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Insecticidal activity guided isolation of palytoxin from a red alga, *Chondria armata*

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ABSTRACT

A red alga, *Chondria armata*, was used as a vermifuge in Japan. Previously, we isolated domoic acids from *C. armata* guided by the insecticidal activity against American cockroaches. Further investigations led to the discovery of a compound one-thousand times more toxic than domoic acid. It showed lethal activity at 5 ng against cockroaches. The insecticidal compound was identified as palytoxin by means of NMR and MS. Careful interpretation of the NMR spectra allowed us to refine the previously reported NMR assignment of palytoxin. Palytoxin has been isolated from various organisms, and it is significant that palytoxin was isolated from marine plants.

Introduction

Chondria armata is a marine red alga found in tropical and subtropical regions, and it was used as a vermifuge in Japan. In 1959, domoic acid was isolated as a vermifugal compound from *C. armata*.[1] We investigated insecticidal compounds in this alga against American cockroaches. As a result, we isolated domoic acid and its congeners.[2,3] In the course of our investigation of insecticidal compounds other than domoic acids, we found an unknown compound with one-thousand times more toxicity than domoic acid. Its molecular weight was more than 2000 Da from analysis of FAB MS spectrum. The NMR spectrum suggested the existence of an olefinic proton neighboring amides and long polyoxygenated carbon chain structures similar to palytoxin.

Palytoxin (PTX) was first isolated from a zoanthid, *Palythoa toxica*, in 1971.[4] The structure of palytoxin (Fig. 1) had been studied for many decades, and its absolute stereochemistry was reported on the basis of organic synthesis in 1982.[5] In 2001, its total NMR signal assignment was achieved.[6] Palytoxin is one of the most toxic compounds, and its intravenous LD_{50} is 150 ng/kg for mice.[4] Palytoxin targets Na^+/K^+-ATPase and transforms it into cation-selective channels, which is inhibited by ouabain.[7] Palytoxin and its congeners were isolated from several marine organisms such as zoanthids, fish, crabs, cyanobacteria, and dinoflagellates,[8] but its original producer has not been clarified. An identification of an original producer of palytoxin is essential for further investigation of its bioconcentration in marine ecosystems.

In this paper, we report the isolation and insecticidal examination of the compound in *C. armata* and its structure determination.

Results and discussion

Isolation of insecticidal compounds in *Chondria armata*

A marine red alga, *Chondria armata*, was collected off the coast of Yakushima Island, Kagoshima prefecture, Japan. The insecticidal examination against American cockroaches was carried out by subcutaneous injection. A water extract of *C. armata* was chromatographed on an ODS column. The methanol fraction showed insecticidal activity, but the lethal effect was different from that of aqueous methanol fractions containing domoic acids. The methanol fraction was further purified using DEAE-cellulose anion-exchange chromatography and two kinds of gel-filtration chromatography with Sephadex LH-20 and TSK G3000S columns. Consequently, we obtained an active compound named CA-II.[9]

CA-II has one-thousand times more insecticidal activity against American cockroaches than domoic acid (Table 1).

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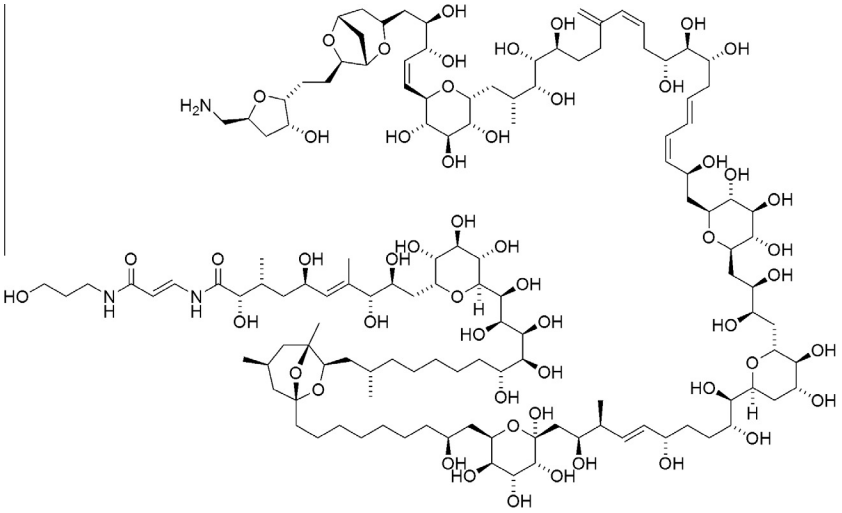

Figure 1. Structure of palytoxin.

Table 1
Insecticidal activity by subcutaneous injection into American cockroaches

Compound	Minimum lethal dose		Relative activity to DDT
	mol/insect	μg/insect	
CA-II	1.8×10^{-12}	0.005	20556
Domoic acid	2.6×10^{-9}	0.8	14
Natural pyrethrin	1.8×10^{-7}	70.0	0.2
γ-BHC	7.0×10^{-8}	20.0	0.5
DDT	3.7×10^{-8}	13.0	1.0

The activity of CA-II was compared with domoic acid from *C. armata*, natural pyrethrin, γ-BHC (Benzenehexachloride, 1,2,3,4,5,6-Hexachlorocyclohexane), and DDT (Dichlorodiphenyltrichloroethane).

Structural analysis

CA-II was isolated as a colorless amorphous solid: $[\alpha]_D^{28}$ +21.6 (*c* 0.266 H_2O); UV(H_2O) λ_{max} 234 nm (ε 23,505) and 264 nm (ε 17,644). The high resolution ESI FT-MS data of CA-II indicated that the molecular formula was $C_{129}H_{223}N_3O_{54}$, which corresponded to that of authentic palytoxin from *Palythoa tuberculosa*, as shown in Figure 2. The retention time for CA-II on a C30 column was also identical with that of palytoxin. The ^{1}H NMR spectrum of CA-II showed a characteristic doublet signal at 7.78 ppm, which was much the same as the signal of the olefinic proton next to the amide bond (Ha) of palytoxin (Fig. 3). The signals from 5.0 to 6.5 ppm were also similar to olefinic protons of palytoxin. Moreover, many signals from 3.0 to 4.0 ppm indicated existence of polyoxygenated carbon chains. UV absorption suggested the presence of an *exo*-methylene and conjugated amide moieties similar to those of palytoxin, and these structures were eventually confirmed by 2D NMR analyses.

To establish the gross structure of CA-II from *C. armata*, extensive 1D and 2D NMR experiments were performed and the spectra were compared to those of authentic palytoxin. The methylene signals next to hemiacetal, H48 and C48, disappeared in the CD_3OD/D_2O solvent system due to deuterium exchange. After changing the solvent from CD_3OD/D_2O to CD_3OH/H_2O, the H48/C48 signal could be detected in the HSQC spectrum.

Proton spin networks were mainly analyzed from DQF-COSY spectra, which allowed us to follow almost all of the proton connectivities (Fig. 4), which were confirmed by TOCSY spectra. For severely signal overlapped regions such as for H12 and H15, the HMQC-COSY method gave well-resolved spectra that were useful for establishment of the spin networks. NOE correlations such as H15/H18 were useful for connection of the partial structure obtained from DQF-COSY and TOCSY. The connectivity around

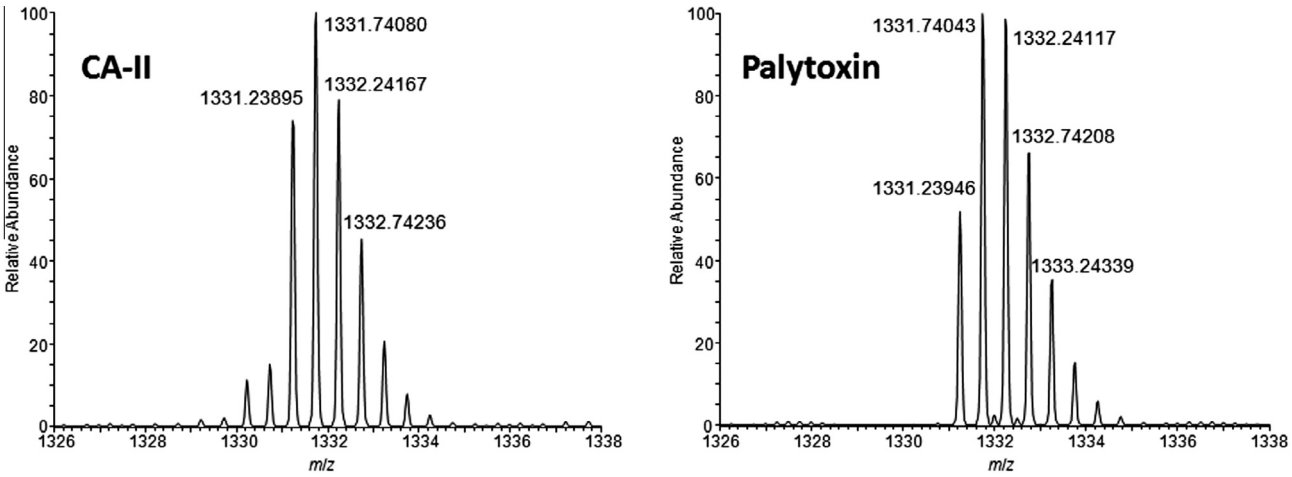

Figure 2. HR ESI FT-MS spectra of CA-II from *C. armata* (left) and authentic palytoxin from *P. tuberculosa* (right).

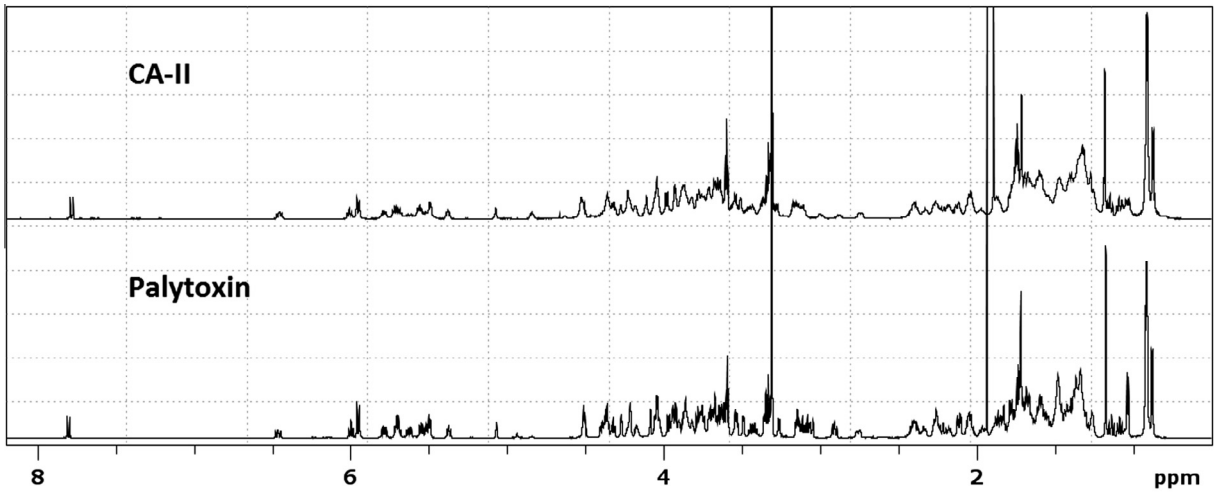

Figure 3. 800 MHz ^{1}H NMR spectra of CA-II from *C. armata* (top) and authentic palytoxin from *P. tuberculosa* (bottom) in CD_3OD with one drop of D_2O.

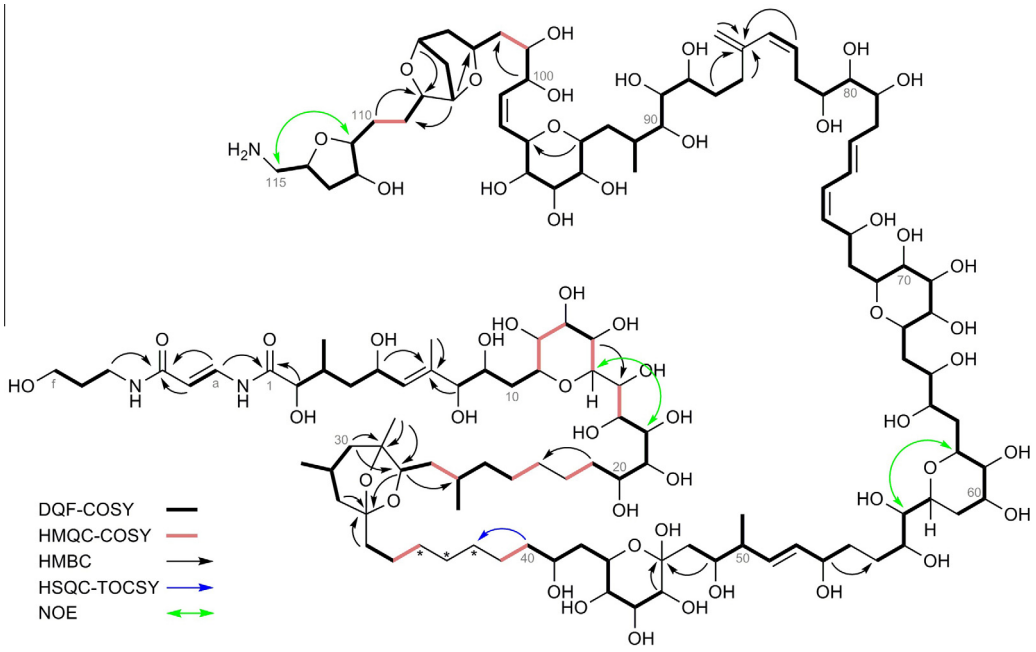

Figure 4. Planar structure and key NMR correlations of CA-II. The marked carbons are interchangeable.

H109 and H110 was confirmed by 1D selective TOCSY experiments at several mixing times.

The index of hydrogen deficiency and the observed carbon signals indicated that CA-II contained ten rings in the molecule. The positions of these rings were deduced from deuterium shift experiments measured in CD_3OH with one drop of H_2O and HMBC experiments. The fifteen oxygenated carbons (C11, C15, C28, C29, C43, C58, C62, C67, C71, C93, C97, C103, C105, C107 and C108) in the middle of the molecule showed deuterium shift below 0.1 ppm, so these carbons were involved in ether linkages. Partial structures from these analyses were connected through HMBC correlations, as shown in Figure 4.

As a result of these NMR analyses, the planar structure of CA-II was successfully determined. The ^{13}C chemical shift of CA-II and authentic palytoxin showed good correspondence, as shown in Table 2. Moreover, detailed analysis of CA-II from *C. armata* and palytoxin from *P. tuberculosa* led us to refine the previously reported signal assignment of palytoxin.[6] In the previous report, TOCSY-based structural analysis presumably led to misunderstanding in the proton spin network analysis. In this report, most of these refinements are based on vicinal proton coupling analysis using the high resolution DQF-COSY method (H18, H19, H21, H23, H57, H58, H109, and H110).

In addition, we compared the optical rotation of CA-II with that of palytoxin. CA-II was optically active with a specific rotation of +21.6° in water. This value was similar to that of authentic palytoxin from *P. tuberculosa* (+22.2°). From the results of NMR methods and optical rotation, we concluded that CA-II was identical to palytoxin.

Conclusion

The present study revealed that the compound from *C. armata* with strong insecticidal activity was identical to palytoxin.

Table 2
Chemical shift data of CA-II from *C. armata* and palytoxin from *P. tuberculosa*

Position	CA-II from *C. armata*			PTX from *P. tuberculosa*			Position	CA-II from *C. armata*			PTX from *P. tuberculosa*		
	^{13}C	^{1}H		^{13}C	^{1}H			^{13}C	^{1}H		^{13}C	^{1}H	
1	175.99	—		175.93	—		60	70.08	3.86		70.20	3.85	
2	75.68	4.11		75.68	4.09		61	76.48	3.16		76.62	3.14	
3	34.65	2.18		34.76	2.18		62	73.11	3.74		73.28	3.75	
3-Me	13.89	0.88		14.01	0.88		63	36.67	1.97	1.69	36.82	1.97	1.71
4	41.63	1.76	1.41	41.77	1.76	1.40	64	71.81	3.68		71.89	3.68	
5	66.58	4.51		66.61	4.51		65	72.10	3.76		72.22	3.76	
6	131.88	5.49		131.82	5.49		66	36.89	2.04	1.54	37.06	2.04	1.54
7	138.26	—		138.32	—		67	77.10	3.46		77.23	3.44	
7-Me	13.09	1.72		13.19	1.72		68	75.93	3.14		76.09	3.11	
8	80.92	3.93		80.91	3.92		69	79.59	3.38		79.79	3.35	
9	72.16	3.82		72.37	3.82		70	75.75	3.11		75.90	3.08	
10	29.29	2.12	1.73	29.18	2.12	1.74	71	77.00	3.43		77.09	3.42	
11	76.09	4.18		76.25	4.17		72	41.35	2.05	1.44	41.58	2.05	1.42
12*3	72.82	3.64		72.94	3.62		73	64.94	4.84		64.97	4.84	
13	75.06	3.56		75.21	3.54		74	133.33	5.38		133.52	5.37	
14	71.62	3.60		71.71	3.60		75	130.09	6.01		130.02	6.00	
15*3	73.73	3.65		73.94	3.65		76	128.83	6.45		128.88	6.46	
16	71.11	4.03		71.33	4.04		77	133.89	5.78		133.92	5.78	
17	71.59	4.04		71.70	4.05		78	38.56	2.41		38.70	2.41	
18*4	71.14	3.78		71.36	3.79		79	71.14	3.93		71.20	3.93	
19*4	73.20	3.54		73.29	3.54		80	76.26	3.28		76.31	3.26	
20	71.05	3.87		71.11	3.88		81	73.01	3.71		73.11	3.70	
21*5	34.95	1.64	1.54	35.06	1.64	1.55	82	34.26	2.74	2.39	34.40	2.76	2.39
22	26.88	1.48	1.36	26.99	1.48	1.37	83	130.14	5.69		130.24	5.69	
23*5	27.33	1.49	1.38	27.43	1.50	1.39	84	132.66	5.95		132.63	5.95	
24	28.41	1.36		28.50	1.37		85	146.61	—		146.78	—	
25	39.68	1.25		39.77	1.26		85'	114.93	5.07	4.94	114.85	5.07	4.94
26	29.64	1.66		29.73	1.68		86	34.24	2.33	2.24	34.33	2.34	2.25
26-Me	19.33	0.92		19.31	0.92		87	33.01	1.73	1.60	33.18	1.73	1.60
27	40.64	1.47	0.91	40.81	1.48	0.91	88	74.13	3.71		74.21	3.71	
28	80.14	3.98		80.16	3.97		89	73.99	3.51		74.04	3.49	
29	82.41	—		82.27	—		90	77.76	3.36		77.85	3.35	
29-Me	20.97	1.18		21.02	1.18		91	33.01	1.88		33.04	1.89	
30	45.62	1.71	1.15	45.76	1.70	1.14	91-Me	15.59	0.91		15.66	0.91	
31	25.48	2.04		25.60	2.05		92	27.75	2.21	1.30	27.87	2.22	1.30
31-Me	21.83	0.92		21.90	0.92		93	74.81	4.05		74.86	4.03	
32	43.65	1.69	1.09	43.79	1.67	1.09	94	72.94	3.67		73.09	3.65	
33	109.28	—		109.20	—		95	74.59	3.63		74.79	3.61	
34	38.51	1.60		38.68	1.60		96	75.96	3.17		76.05	3.15	
35	23.92	1.41		24.01	1.42		97	69.61	4.32		69.70	4.32	
36*2	30.87	1.33		31.01	1.34		98	132.33	5.56		132.46	5.55	
37*2	30.87	1.33		31.01	1.34		99	135.37	5.72		135.28	5.71	
38*2	30.76	1.32		30.88	1.34		100	71.91	4.36		71.87	4.37	
39	31.23	1.35		31.34	1.37		101	71.69	3.68		71.78	3.68	
40	39.08	1.48		39.25	1.48		102	40.09	1.59		40.26	1.59	
41	69.20	3.78		69.26	3.77		103	68.41	4.22		68.34	4.22	
42	39.27	1.86	1.44	39.44	1.87	1.44	104	40.39	1.75	1.39	40.59	1.74	1.38
43	64.79	4.39		64.86	4.40		105	76.13	4.53		76.15	4.51	
44	73.79	3.65		73.94	3.63		106	36.76	1.85	1.79	36.87	1.82	1.78
45	74.13	3.96		74.33	3.94		107	79.59	4.23		79.62	4.21	
46	68.15	3.68		68.30	3.67		108	82.75	4.36		82.72	4.36	
47	101.10	—		101.28	—		109*7	32.19	1.49		32.36	1.48	
48	41.88*1	1.82*1		41.96	1.83		110*7	26.52	1.77	1.65	26.61	1.79	1.67
49	72.28	3.94		72.41	3.95		111	83.69	3.88		83.89	3.90	
50	43.99	2.27		44.12	2.26		112	73.26	4.27		73.25	4.27	
50-Me	16.73	1.03		16.59	1.04		113	39.61	2.11	1.87	39.87	2.11	1.87
51	134.52	5.61		134.44	5.63		114	75.87	4.35		74.81	4.38	
52	134.75	5.51		134.86	5.50		115	45.28	2.97	2.86	44.93	3.06	2.91
53	74.05	4.06		74.08	4.05		a	134.65	7.78		134.81	7.80	
54	34.84	1.78	1.61	34.98	1.78	1.61	b	106.90	5.95		106.76	5.95	
55	27.62	1.69	1.46	27.81	1.70	1.47	c	169.67	—		169.67	—	
56	72.95	3.75		73.12	3.75		d	37.40	3.33		37.41	3.33	
57*6	74.04	3.89		74.19	3.86		e	33.14	1.74		33.34	1.74	
58*6	72.79	3.85		72.79	3.86		f	60.35	3.60		60.41	3.59	
59	32.92	2.27	1.67	33.07	2.27	1.67							

CA-II and palytoxin were dissolved in CD_3OD with one drop of D_2O. Chemical shifts were determined in ppm for both ^{1}H and ^{13}C. Residual methanol signals were used as internal chemical shift references of 3.31 ppm in ^{1}H and 49.00 ppm in ^{13}C NMR spectra, respectively. The connectivity of H48 to C48 (*1) was assigned by CD_3OH with one drop of H_2O. The marked rows *2 show a group of carbons which are interchangeable each other, and the marked rows from *3 to *7 show the refinement of signal assignment from the previous report.[6]

Palytoxin and its congeners have been found in several organisms, and it is significant that palytoxin was isolated from marine plants. This finding will contribute to the investigation of the bioconcentration in marine ecosystems of palytoxin.

Additionally, we refined the NMR signal assignment of palytoxin. The exact signal assignment of palytoxin plays an important role in the structural determination of unknown palytoxin congeners using NMR methods.

Experimental methods

Isolation of CA-II from *Chondria armata*

The red algae were collected off the coast of Yakushima Island during July and August, dried in the dark, and stored at −20 °C until extraction. The dried plant (400 g) was crushed and extracted with 4 L of water below 10 °C. After removal of solid residue by centrifugation, the water extract was passed through a DEAE-cellulose column (10 × 5 cm, OH^- form) and washed with 0.9 L of water. The flow-through fraction was charged on a LiChroprep RP-18 column (5 × 5 cm). After washing with 1 L of water, 1 L of 25% methanol and 1 L of 50% methanol, compounds were eluted with 1.9 L of methanol and 2.5 L of 2% acetic acid containing methanol. The methanol (methanol and 2% acetic acid containing methanol) fractions were concentrated under reduced pressure below 40 °C. The resulting residue was applied to a Sephadex LH-20 column (2.6 × 190 cm) equilibrated with 0.5% acetic acid containing 50% methanol before use, separated into fractions of 15 mL each and applied to insecticidal activity evaluation.

The toxic fractions were concentrated under reduced pressure below 40 °C and lyophilized. The samples were dissolved in water and applied to a TSK G3000S column (1 × 7.5 cm). After washing with water, the toxin was eluted with 50% ethanol and fractionated into 2 mL aliquots. CA-II (1.02 mg) was obtained as colorless amorphous powder.

Domoic acid for insecticidal examination was isolated from *C. armata* as described in the previous report.[2]

Insecticidal examination against American cockroaches

American cockroaches, *Periplaneta americana* L., were reared on an artificial diet at 25 °C and 60% relative humidity, under short-day photoperiods (12 L: 12 D). Insecticidal examination was carried out as described in the previous report.[3]

FAB MS experiments

The fast atom bombardment mass spectrometry (FAB MS) analysis was carried out on a JMS-HX110 mass spectrometer (JEOL Ltd, Japan) equipped with a FAB gun using xenon gas. The source was operated at an accelerating voltage of 5.0 kV and the FAB gun at an accelerating voltage of 6.0 kV in positive ion mode. The samples were dissolved in H_2O and mixed with glycerol. The spectra were run in a mass range from m/z 100 to 3500.

LC–MS experiments

An Orbitrap Elite FT mass spectrometer (Thermo Fisher Scientific, Inc., USA) was used for HR ESI FT-MS data acquisition. The mass spectrometer was coupled with a Shimadzu Prominence high performance liquid chromatograph (Shimadzu Corporation, Japan). Data analyses were carried out with both Thermo Xcalibur 2.2 (Thermo Fisher Scientific, Inc., USA) and LabSolutions version 5.53 (Shimadzu Corporation, Japan). The samples were separated on a reverse phased column, Develosil C30-UG-3 2.0 i.d. × 100 mm (Nomura Chemical Co., Ltd, Japan) with a gradient solvent system: 10% B for 0–2 min, 10–50% B for 2–22 min, 50–100% B for 22–23 min, 100% B for 23–25 min, 100–10% B for 25–26 min, 10% B for 26–30 min, where solvent A was 0.1% acetic acid and solvent B was 0.1% acetic acid containing acetonitrile. The flow rate was set to 0.2 mL/min. The analytical condition of the mass spectrometer was full MS scan range m/z 300–3000 with mass resolution of 30,000 at m/z 400 in positive ion mode.

CA-II; HRESIMS (m/z): m/z 1331.2390 $[M+2H-H_2O]^{2+}$ (calcd for $C_{129}H_{223}N_3O_{54}$ 1331.2418); t_R 15.20 min.

Authentic palytoxin; HRESIMS (m/z): m/z 1331.2395 $[M+2H-H_2O]^{2+}$ (calcd for $C_{129}H_{223}N_3O_{54}$ 1331.2418); t_R 15.19 min.

NMR experiments

NMR spectra were acquired on a Bruker AVANCE III HD 800 spectrometer equipped with a 5-mm TCI cryogenic probe and Z-axis gradient (Bruker Biospin AG, Switzerland). All spectra were measured at 298 K. Wilmad 5 mm NMR tubes were used. CA-II from *Chondria armata* (4 mg) and palytoxin from *Palythoa tuberculosa* (2.5 mg) were dissolved in 0.6 mL of CD_3OD or CD_3OH with one drop of D_2O or H_2O. Chemical shifts were reported in parts per million (ppm) relative to residual methanol peaks (δ_H 3.31 and δ_C 49.00 for CHD_2OD or CHD_2OH).

Standard Bruker pulse sequences were employed. Solvent suppression by presaturation was used when required. All NMR data reported in this article were derived from DQF-COSY, TOCSY, NOESY, HSQC, HMBC, HSQC-TOCSY, and HMQC-COSY experiments. Data analyses were carried out with Bruker TopSpin 3.2 software (Bruker Biospin AG, Switzerland).

The ^1H and ^{13}C chemical shifts of CA-II and palytoxin matched within 0.04 ppm (^1H) and 0.25 ppm (^{13}C) except for the signals near the terminal amine. The slight differences in the 1D ^1H NMR spectra of these were likely caused by signals of contaminated palytoxin congeners.

UV–vis and optical rotation

UV–vis spectra were measured on a V-650 UV–Vis spectrophotometer (JASCO Corporation, Japan). A 10 mm quartz cell was used. CA-II and authentic palytoxin were dissolved in water. The concentrations of CA-II and authentic palytoxin were 39.7 μM and 30.7 μM, respectively.

Optical rotations were measured in water on a DIP-1000 digital polarimeter (JASCO Corporation, Japan). The concentration of CA-II and authentic palytoxin were 0.266 g/dL and 0.137 g/dL, respectively. The optical rotation values were averaged from five measurements.

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